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Identification of *opsA*, a Gene Involved in Solute Stress Mitigation and Survival in Soil, in the Polycyclic Aromatic Hydrocarbon-Degrading Bacterium *Novosphingobium* sp. Strain LH128

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The aim of this study was to identify genes involved in solute and matric stress mitigation in the polycyclic aromatic hydrocarbon (PAH)-degrading *Novosphingobium* sp. strain LH128. The genes were identified using plasposon mutagenesis and by selection of mutants that showed impaired growth in a medium containing 450 mM NaCl as a solute stress or 10% (wt/vol) polyethylene glycol (PEG) 6000 as a matric stress. Eleven and 14 mutants showed growth impairment when exposed to solute and matric stresses, respectively. The disrupted sequences were mapped on a draft genome sequence of strain LH128, and the corresponding gene functions were predicted. None of them were shared between solute and matric stress-impacted mutants. One NaCl-affected mutant (i.e., NA7E1) with a disruption in a gene encoding a putative outer membrane protein (OpsA) was susceptible to lower NaCl concentrations than the other mutants. The growth of NA7E1 was impacted by other ions and nonionic solutes and by sodium dodecyl sulfate (SDS), suggesting that *opsA* is involved in osmotic stress mitigation and/or outer membrane stability in strain LH128. NA7E1 was also the only mutant that showed reduced growth and less-efficient phenanthrene degradation in soil compared to the wild type. Moreover, the survival of NA7E1 in soil decreased significantly when the moisture content was decreased but was unaffected when soluble solutes from sandy soil were removed by washing. *opsA* appears to be important for the survival of strain LH128 in soil, especially in the case of reduced moisture content, probably by mitigating the effects of solute stress and retaining membrane stability.

Microbial degradation is considered a major process that contributes to the removal of polycyclic aromatic hydrocarbons (PAHs) in soil (1). Sphingomonads are an important group of bacteria involved in the biodegradation of PAHs in PAH-contaminated environments. Sphingomonad bacteria are widely distributed in soil, water, and sediments, and various PAH-degrading sphingomonads have been isolated from PAH-contaminated environments (2, 3). Their importance for PAH biodegradation in soil has been shown by using ^{13}C-labeled PAH substrates in lipid- or DNA-based stable isotope probing approaches (4, 5). Sphingomonads have also been associated with the biodegradation of various organic xenobiotics other than PAHs (6–9).

Successful bioremediation requires knowledge about the environmental factors that determine the activity and survival of pollutant-degrading microorganisms in the contaminated habitat. The nature of these factors can be physicochemical or biological. Physicochemical factors include moisture content, temperature, radiation, pH, osmotic pressure, presence of toxic chemicals, and nutrient limitation. Biological factors are related to the presence of predators and competitors (10–12). Suboptimal conditions can lead to stress and suboptimal catabolic activity, the consequence of which can be poor pollutant degradation rates (13). One of the most important environmental factors that influence the survival and activity of bacteria in soil is the water potential (14). The two major components of the soil water potential are the solute and the matric potentials. The solute potential decreases with increasing concentrations of solutes, such as salts and nonionic solutes, while the matric potential describes the interaction of water with surfaces and interfaces, including colloidal and solid particles (15). In soil, both are frequently subjected to periodic fluctuations due to changes in natural environmental conditions and/or to anthropogenic activities. Weather-bound drought-wet cycles lead to changing solute and matric potentials (16). In addition, a reduction in water potential can occur through the input of solutes from industrial and agricultural activities, such as irrigation with water of poor quality and the use of salt for the deicing of roads in winter (17, 18). The latter leads to the input of NaCl in the upper soil layers, in particular, in urban environments and near highways, which are also exposed to significant PAH pollution (18, 19). Moreover, uptake of water by plants can result in a local increase in the solute concentration around the plant roots, lowering the water potential for microorganisms (17). To study the impact of changes in solute potential and, hence, solute stress on bacterial physiology under laboratory conditions, increasing concentrations of ionic solutes like NaCl or nonionic solutes like sucrose are used in the growth medium, while to study the impact of matric stress, nonpermeating high-molecular-weight polyethylene glycol (PEG) is often added (20, 21).

The ability of microorganisms to adapt to and withstand stress

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situations is governed by the expression of specific stress response genes (11). The majority of genes that are important for activity/survival under stress conditions, such as σ factors (22, 23), have been identified in pathogenic bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa* (24–27). There is little information about the ecological role of the identified genes in a natural environment such as soil, especially for pollutant-degrading bacteria (28). For instance, there is no knowledge of the gene functions involved in matric and solute stress resistance in sphingomonads, although this is important to understand the adaptive strategies of the genus *Sphingomonas* for bioremediation in soil. Differential genome-wide gene expression in response to solute and matric stress was reported for the dibenzofuran-degrading organism *Sphingomonas wittichii* RW1 (29), and differential gene expression in response to solute stress was reported for the PAH-degrading bacterium *Novosphingobium* sp. strain LH128 (30), but in neither case was functional analysis performed for the differentially expressed genes. More recently, genome-wide transposon scanning was used to identify essential genes in strain RW1 under conditions of solute stress (31).

In this study, plasposon mutagenesis was used to identify genes involved in coping with solute and matric stress in the PAH-degrading organism *Novosphingobium* sp. LH128. A mutant library of the strain was constructed and was screened for mutants that showed growth impairment in the presence of solute or matric stress. As in other studies that have addressed the effect of solute and matric stress on bacterial physiology and activity, NaCl and PEG 6000 were used in the screening procedure to generate solute and matric stress, respectively (16, 32–35). The relevance of selected identified gene functions for survival and for the PAH degradation activity of LH128 was investigated in PAH-contaminated soils with various moisture contents.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture methods. *Novosphingobium* sp. strain LH128, formerly denominated *Sphingomonas* sp. strain LH128, was isolated from PAH-contaminated soil and utilizes phenanthrene as the sole source of carbon and energy (2). According to a phylogenetic analysis similar to that performed by Aylward et al. (36) but based on an alignment of concatenated amino acid sequences (~10,200 residues) from a smaller subset of 25 representative proteins with housekeeping function from 30 different sphingomonad strains with known (draft) genome sequences, the strain was renamed *Novosphingobium* sp. LH128, as previously suggested by its 16S rRNA gene sequence (37). Strain LH128 was routinely grown at 27°C in R2A medium (38) or on R2A agar plates. LH128 mutants were screened on selective phosphate-buffered minimal medium (MM) (39). *E. coli* BW20767 harboring pRL27 carrying the Tn*5*-based plasposon (containing the *tetA* promoter, the *tnp* transposase, the *aph* gene encoding kanamycin resistance, and the *oriR6K* replicon) (40) and *E. coli* DH5α λ*pir* (containing the *tetM* gene encoding tetracycline resistance and a derivative of phage λ carrying a cloned copy of the *pir* gene) (41) were grown at 37°C in Luria broth (LB) containing tryptone (10 g), yeast extract (5 g), and NaCl (5 g) (Difco) or on LB agar plates containing 50 mg liter^{-1} kanamycin (Km).

Transposon mutagenesis. pRL27 was introduced into strain LH128 by conjugation from *E. coli* strain BW20767. *E. coli* and LH128 were grown to an optical density at 600 nm (OD_{600}) of 0.9 (Spectronic 20; Milton Roy). Donor cells were washed, and both cultures were adjusted to the same turbidity (OD_{600}, 0.9) in LB medium. The strains were mixed at a ratio of 1:1 (2 ml of the donor and 2 ml of the recipient) for conjugation. The suspension was centrifuged for 5 min at 5,000 × *g* in a Beckman Coulter Allegra 25R centrifuge (Analis SA, Belgium), and the cell pellet was resuspended in 50 μl sterile saline solution (0.9% NaCl) and spotted onto LB agar plates that were incubated at 27°C for 16 h. The cells were resuspended in 1 ml saline solution, after which 200 μl of each suspension was spread on MM agar plates containing 2 g liter^{-1} glucose and Km (50 mg liter^{-1}). Colonies that appeared after 3 days were transferred to 96-well microtiter plates containing 200 μl MM plus 2 g liter^{-1} glucose and Km (50 mg liter^{-1}) per well. The plates were incubated overnight at 27°C while shaking at 160 rpm and then stored at −80°C after adding 100 μl of 50% glycerol solution.

Screening for mutants defective in matric or solute stress resistance. All mutants were replica plated onto square agar plates (12 by 12 by 1.7 cm) containing agarose-solidified MM with 2 g liter^{-1} glucose, Km (50 mg liter^{-1}), agarose (1.5%), and 450 mM NaCl or into microtiter plates containing 200 μl per well of liquid MM with 2 g liter^{-1} glucose, Km (50 mg liter^{-1}), and 10% (wt/vol) PEG 6000. The mutants were simultaneously replica plated on or in the same medium without NaCl or PEG 6000 as controls. Mutants affected in matric stress mitigation were identified in liquid medium instead of on solid medium since agarose does not solidify in the presence of 10% PEG. Microtiter plates or agar plates were visualized for decreased culture turbidity, the absence of growth, or a reduced colony size in the presence of the applied stresses compared to that for growth under nonstress conditions and compared to that for the growth of wild-type strain LH128 after 3 to 5 days of incubation at 27°C. Candidate mutants were purified, and their phenotypes were confirmed.

Southern blot hybridization. Selected mutants were grown overnight in MM containing 2 g liter^{-1} glucose and Km (50 mg liter^{-1}) until the OD_{600} reached 0.7 (Spectronic 20; Milton Roy). One milliliter of the cultures was centrifuged for 1 min at 10,000 × *g*, and genomic DNA was isolated from the pelleted cells using a Gentra Puregene bacterial kit (Qiagen Benelux B.V.). One microgram DNA was digested for 3 h at 37°C in a 20-μl reaction mixture containing 2 μl of NEBuffer 3, 1 μl of bovine serum albumin, and 13 U of BamHI (New England Biolabs GmbH, Germany), which does not cut within the transposon sequence. The digested genomic DNA was separated on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer and blotted onto an Amersham Hybond-N membrane (GE Healthcare Benelux B.V.). A part of the Km resistance gene from pRL27 was amplified by PCR in the presence of digoxigenin-labeled nucleotides (Roche Diagnostics GmbH, Germany), using primers Km377F (5′-TGT TCCTGCGCCGGTTGCAT-3′) and Km722R (5′-GGAGAAAACTCAC CGAGGCA-3′), and used as a probe. Nucleic acids were hybridized for 16 h at 62°C in hybridization buffer containing 25% (vol/vol) saline sodium citrate (SSC) buffer (17.6 g liter^{-1} NaCl, 8.8 g liter^{-1} trisodium citrate dihydrate), 20% (vol/vol) formamide, 2% (vol/vol) blocking reagent (1.75 g liter^{-1} NaCl, 2.32 g liter^{-1} maleic acid, 2.2 g liter^{-1} blocking powder), 0.1% (wt/vol) Na-lauroylsarcosine, and 0.02% (wt/vol) SDS, as described previously (42). Washing was done twice for 5 min each time in low-stringency buffer containing 10% SSC and 0.1% SDS at room temperature and then twice for 15 min each time in high-stringency buffer containing 0.1× SSC and 0.1% SDS at 68°C. Detection was performed with an alkaline phosphatase-coupled antidigoxigenin antibody using CDP-Star chemiluminescent substrate (Roche Diagnostics GmbH, Germany). Membranes were then exposed to X-ray film (Kodak) for 5 min.

Sequencing and analysis of the plasposon flanking regions. BamHI-digested genomic DNA was self-ligated overnight at 16°C in a 100-μl reaction mixture containing 500 ng of digested DNA, 10 μl of 10× ligation buffer, and 1.5 U of T4 DNA ligase (Promega Benelux B.V.). After addition of 1 ml of *n*-butanol, the ligation mixture was centrifuged at 15,000 × *g* for 15 min. The pellet was washed twice with 70% ethanol, dried at room temperature, and dissolved in 10 μl of water. Five microliters of the purified ligation product (250 ng DNA) was transformed to competent *E. coli* DH5α λ*pir* cells by heat shock at 42°C for 90 s. The mixture was incubated at 37°C for 1 h in SOC medium (Invitrogen) and then plated onto LB agar plates containing Km (50 mg liter^{-1}). The plasposon with its flanking DNA was isolated from the transformants using a Wizard SV genomic DNA purification kit (Promega Benelux B.V.). The

regions flanking the insertion sites were sequenced using primers complementary to the ends of the plasposon, i.e., primers tpnRL17-1 (5′-AACA AGCCAGGGATGTAACG-3′) and tpnRL13-2 (5′-CAGCAACACCTTC TTCACGA-3′) (40), by means of BigDye Terminator cycle sequencing, as recommended by the manufacturer (Life Technologies Benelux B.V.). The sequences were mapped on the annotated draft genome sequence of LH128 (DDBJ/EMBL/GenBank accession number ALVC00000000; first version, DDBJ/EMBL/GenBank accession number ALVC01000000). The similarity of the deduced protein sequences to protein-coding sequences in NCBI was determined by using the BLASTP algorithm, and homology with the known proteins in the Wellcome Trust Sanger Institute database was determined by using protein family (Pfam) matches for additional verifications.

Matric potential, solute dose, and SDS response assay. The growth rates of wild-type strain LH128 and mutants on glucose were measured in triplicate in 75-ml test tubes (25 by 200 mm) containing 20 ml MM with 2 g liter^{-1} glucose and increasing concentrations (100, 200, 300, 400, 600 mM) of NaCl, LiCl, Na_2SO_4, or sucrose to evaluate the effect of solute stress and increasing concentrations of PEG 6000 (0.1, 1, 5, 10, 20% [wt/vol]) to evaluate the effect of matric stress. The concentrations of solutes reported above reduced the water potential by 0.49, 0.74, 1.48, 2.22, and 2.97 MPa, respectively, in the case of NaCl or LiCl; by 0.74, 1.1, 2.23, 3.34, and 4.45 MPa, respectively, in the case of Na_2SO_4; and by 0.25, 0.37, 0.74, 1.11, and 1.48 MPa, respectively, in the case of sucrose, as calculated according to the van't Hoff equation (29, 43). The reduction in water potential by 0.1, 1, 5, 10, and 20% PEG 6000 was calculated to be 4.1×10^{-4}, 5.01×10^{-3}, 0.045, 0.18, and 1.38 MPa, respectively, according to previously described methods (43). Increasing concentrations (0.05, 0.1, 0.5 g liter^{-1}) of SDS were used to evaluate the structural role of OpsA in outer membrane stability. The strains were pregrown in MM with 2 g liter^{-1} glucose, harvested in the exponential growth phase (OD_{600}, 0.6; Spectronic 20; Milton Roy), washed twice, and suspended in MM to an OD_{600} of 0.2. Ten milliliters of this suspension was pelleted and used to inoculate the test tubes with a final OD_{600} of 0.1. The OD_{600} was followed over time, and growth rates were calculated from the slope of the growth curves.

Complementation of mutant NA7E1. The *opsA* gene with a 198-bp putative promoter region was amplified from wild-type strain LH128 using forward primer OpsAFKpnI (5′-AGAGGTACCTCGGTTTGACGTG CGCAAG-3′) containing a KpnI restriction site (underlined) and reverse primer OpsARHindIII (5′-AGAAAGCTTCACCGATCCGACGTCTTA C-3′) containing a HindIII restriction site (underlined). The fragment was purified from agarose, digested with KpnI and HindIII, and ligated in the KpnI/HindIII-digested vector pME6012. The recombinant plasmid, designated pME6012OpsA, was transformed into *E. coli* S17-1 λ*pir* as described above. pME6012OpsA was transferred from *E. coli* S17-1 λ*pir* to LH128 mutant NA7E1 by conjugation. Transconjugants were selected on MM agar plates containing 2 g liter^{-1} glucose and tetracycline (5 mg liter^{-1}).

Soil microcosm experiments. Three different soils were used, i.e., a haplic luvisol soil designated Ter Munck (TM), a historically PAH-impacted soil designated Sobeslav, and commercial quartz sand. The characteristics of the soils are presented in Document S1 in the supplemental material. The experiment with quartz sand was conducted using both washed and nonwashed sand. Washing was done 5 times by mixing 100 g of sand with 500 ml of Milli-Q water, followed by decantation of the water. Both the washed sand and the nonwashed sand were dried by incubating at 105°C for 24 h. Before use, all soils were passed through a 2-mm-pore-size sieve and homogenized. Nonsterile soil portions with the water content at the water-holding capacity (WHC) and 25% of the WHC were prepared as follows. The water content of the sieved soils was first adjusted so that it was to close to their WHC using Milli-Q water, taking into consideration the volume of the inoculum to be added later (see below). Afterwards, a portion of the soils in which the water content was adjusted to the WHC was spread as a thin layer onto a clean sterile aluminum tray and air dried with physical homogenization until the water content reached close to 25% of the WHC, taking into consideration the volume of inocula to be added later (see below). Sterile soils with water contents at the WHC and 25% of the WHC were prepared as follows: the soils were dried in an oven at 105°C for 24 h and sterilized three times by autoclaving at 121°C for 15 min each time, followed by incubation at 20°C for 2 days. The sterility was confirmed by plating the soil suspensions on R2A agar plates.

One gram (dry weight) of sterile/nonsterile soil with a moisture content of either the WHC or 25% of the WHC was added into sterile 15-ml glass Pyrex tubes with screw caps. Microcosms were prepared in triplicate for each time point to be analyzed and for each experimental condition. The soil in the tubes was spiked with 50 μl of a phenanthrene dissolved in acetone solution (10 g phenanthrene liter^{-1}), leading to a final concentration of 500 mg kg^{-1} soil. Acetone was left to evaporate under a laminar flow hood for 15 h, and the soils were homogenized gently on a multitube vortexer for 30 s (VX-2500 multitube vortexer; VWR). The microcosms were inoculated with rifampin (Rif)-resistant (Rif^r) variants of the tested mutants or the wild-type strain. Those variants were obtained by streaking the strains on MM containing 2 g liter^{-1} of glucose and Rif (50 mg liter^{-1}) and purification of single colonies on the same medium. Rif^r variants were checked for their matric and solute stress phenotypes and phenanthrene degradation phenotype and then grown in an MM culture containing 2 g liter^{-1} of glucose and Rif (50 mg liter^{-1}). The cultures were washed once, resuspended in 0.01 M $MgSO_4$, and used for inoculating the soil microcosms at a final density of 1×10^9 CFU per gram of soil. The vials were capped and incubated without shaking at 20°C for 4 h, 10 days, and 20 days. At each time point, triplicate tubes were sacrificed for determining the numbers of CFU and the residual phenanthrene concentration. To determine the numbers of CFU, 5 ml of 0.01 M $MgSO_4$ was added to each tube and the tubes were shaken end over end for 30 min. The soil particles were allowed to sediment for 15 min. Two 200-μl portions of the suspension were taken from each tube and serially diluted in 0.01 M $MgSO_4$ in microtiter plates, from which 5 μl was spotted in triplicate on square MM agar plates containing 2 g liter^{-1} of glucose and Rif (50 mg liter^{-1}). The detection limit was 1,000 CFU per g of soil. Rif^r cells recovered from the soil microcosm were checked for the relevant phenotypes by growing about 20 colonies on MM agar plates containing 2 g liter^{-1} glucose with and without 450 mM NaCl or 10% PEG 6000, with and without Km (50 mg liter^{-1}), and with and without Rif (50 mg liter^{-1}) and on agar plates with and without phenanthrene. In all cases, the phenotypes of the recovered colonies agreed with those of the inoculum strains. To determine the residual phenanthrene concentrations in the tubes, the soil suspension in $MgSO_4$ was centrifuged for 10 min at 3,070 × *g*, after which phenanthrene was extracted from the soil pellet by means of a hexane-acetone (4:1 ratio) solution. Briefly, 2.5 ml of a hexane-acetone solution was added, the tubes were vortexed on a multitube vortexer for 2 min and centrifuged at 3,070 × *g* for 10 min, and the supernatant was collected in a 1.5-ml glass vial. The pelleted soil was extracted a second time using the same procedure, and both extracts were pooled. The phenanthrene concentration in the extracts was analyzed by high-performance liquid chromatography (HPLC) from triplicate tubes as described previously (44) using a Platinum EPS C_{18} 100A 3U column and a UV detector set at 254 nm with a mobile phase consisting of Milli-Q H_2O and CH_3CN (25/75%).

Statistical analysis. Where appropriate, significant differences between wild-type strain LH128 and the mutant or between stress conditions were assessed by means of multiple pairwise comparisons using analysis of variance in combination with a *post hoc* Tukey's honestly significant difference (HSD) test at a confidence level of 95% implemented in SPSS (version 11.5) software.

RESULTS

Screening and characterization of mutants. To select an appropriate concentration of NaCl or PEG for mutant identification, the growth of the wild-type LH128 strain on glucose with different

concentrations of NaCl or PEG 6000 was first examined. NaCl concentrations ranging from 50 to 600 mM and PEG 6000 concentrations ranging from 5 to 10% resulted in increased lag phases and slightly decreased growth rates of strain LH128 on glucose compared to those of the control cultures. However, at all tested salt and PEG concentrations, strain LH128 ultimately achieved the same OD_{600} as it did in NaCl- or PEG 6000-free MM (30; T. T. Fida and D. Springael, unpublished results). Concentrations of NaCl and PEG 6000 that clearly affected LH128 growth (i.e., a ca. 20% growth rate reduction in the exponential phase and an increase in the lag time of about 24 h), i.e., 450 mM NaCl and 10% PEG 6000, were chosen for identifying mutants within a mini-Tn*5* plasposon mutant library of strain LH128 affected in growth on glucose in the presence of salt and matric stress, respectively. A total of 5,000 mutants (which, when assuming random insertions, would statistically result in 94% of the draft annotated open reading frames being hit once by a plasposon) were obtained. Of these, 11 and 14 mutants showed impaired growth (an absence of growth or a reduced colony size on agar plates and an absence of growth or a reduced growth rate in liquid medium) on glucose compared to the wild-type strain when exposed to either solute or matric stress, respectively. Southern blot hybridization of digested genomic DNA showed that a single copy of the plasposon had integrated at different positions of the genome in the different mutants (see Fig. S1 in the supplemental material). For all selected mutants, the sequences flanking the transposon sites were mapped on a draft genome sequence of strain LH128. All the insertions were within predicted open reading frames (ORFs), except in mutants PE37C12 and NA40B6, in which the plasposon was inserted within an intergenic region or putative promoter region of a downstream gene (coding for a hypothetical protein) (see Fig. S2 in the supplemental material). An overview of the disrupted putative gene functions is shown in Table 1.

Analysis of dose-response of selected mutants toward matric and solute stress. The effect of increasing PEG 6000 or NaCl concentrations on the growth of selected mutants on glucose was determined. The selected mutants were mutants PE11E1, PE26E12, PE38B5, PE49E6, NA7E1, NA36D9, and NA43C6. These mutants were chosen as they differed in their growth patterns (reduced growth rate or no growth at all in the presence of 450 mM NaCl or 10% PEG) and were affected in genes whose deduced protein sequence showed homology to genes with a known and relevant function, i.e., genes involved in transport and membrane proteins (Table 1). All examined matric stress-affected mutants showed reduced growth rates in the presence of PEG 6000 at concentrations above 10% compared to that of the wild-type strain, whereas all solute stress-affected mutants showed reduced growth rates in the presence of NaCl at concentrations above 400 mM (see Fig. S3 in the supplemental material), with the exception of mutant NA7E1, which exhibited reduced growth at 150 to 200 mM NaCl and did not grow in the presence of NaCl at concentrations above 300 mM (Fig. 1A and B; see Fig. S4 in the supplemental material) even after 48 h (see Fig. S4 in the supplemental material).

Mutant NA7E1, which was the most severely impacted by the solute stress, was further characterized. The gene whose function was disrupted in this particular mutant encoded an outer membrane protein-like protein. The location of the plasposon insertion together with the surrounding genes is shown in Fig. 2. The nearest upstream and downstream ORFs are distantly located, and no direct operon structure with the disrupted gene could be noticed. Moreover, neither the upstream gene, which encodes a hypothetical protein, nor the downstream gene, which encodes a putative queuosine biosynthesis QueC ATPase, has previously been associated with solute stress. The gene was named *opsA* for the outer membrane protein of sphingomonads. To verify that the effects observed with NA7E1 were caused by the loss of the *opsA* gene, a recombinant pME6012 plasmid variant (pME6012OpsA) containing *opsA* and its 198-bp upstream region was constructed and introduced into NA7E1. The mutant containing the recombinant plasmid was examined for growth on glucose in the presence of 450 mM NaCl. The growth of NA7E1 carrying pME6012OpsA in the presence of 450 mM NaCl was similar to that of the wild-type strain (Fig. 3). This showed that the mutation in *opsA* itself affected the growth of LH128 in the presence of solute stress, that no other spontaneous mutations had occurred, and that the observed phenotype was not due to polar effects of the insertion. The growth of mutant NA7E1 on glucose was further determined in the presence of the permeating solutes Na_2SO_4, LiCl, and sucrose and of the nonpermeating compound PEG 6000. There was no difference in growth between NA7E1 and wild-type strain LH128 in the presence of the concentrations of PEG 6000 tested (data not shown). On the other hand, LiCl, Na_2SO_4, and sucrose caused effects similar to those caused by NaCl (Fig. 1). LiCl or Na_2SO_4 at a concentration of 200 mM resulted in about a 90% reduction in the growth rate of the mutant (Fig. 1D and F), whereas a 10% reduction in the growth rate of wild-type strain LH128 was seen (Fig. 1C and E). Sucrose at 400 mM reduced the growth rate of the mutant by 95% (Fig. 1H), but it reduced that of wild-type strain LH128 by only 35% (Fig. 1G). Since OpsA might be an important protein for the outer membrane structure, the growth of NA7E1 was compared with that of the wild type under conditions with 2 g $liter^{-1}$ glucose in the presence of different concentrations of SDS. The growth of NA7E1 was impaired from an SDS concentration of 0.05 g/liter, but that of the wild type was impaired only beyond 0.1 g/liter SDS (Fig. 4).

Survival and phenanthrene-degrading activity of LH128 mutants in soil. Mutants with a disruption in the major facilitator superfamily (MFS) transporter (PE11E1), the Fur family Fe^{2+}/Zn^{2+} uptake regulator (PE26E12), OpsA (NA7E1), and the Na^+/solute symporter (NA43C6) were further examined for survival and phenanthrene degradation activity in sterile and nonsterile soil microcosms containing TM soil amended with phenanthrene. The moisture content of the soil was adjusted to either its WHC or 25% of its WHC. The lower moisture content, we assumed, would possibly invoke additional solute stress. The populations of most of the mutants developed similarly to the LH128 wild-type population, and the rates of phenanthrene degradation in the soil were indistinguishable (data not shown). For all mutants as well as the wild-type strain, the numbers of CFU per g of soil declined drastically within 4 h after inoculation in both sterile and nonsterile TM soils. This decrease was followed by an increase in the numbers of CFU and concomitant phenanthrene degradation for both the wild type and most of the mutants, regardless of the moisture content. The exception was mutant NA7E1, which reached a lower final population size (as determined from the numbers of CFU per g of soil) in sterile TM soil at the WHC than the wild-type strain (Fig. 5A). The decrease in the population size of mutant NA7E1 was concomitant with incomplete and slower phenan-

TABLE 1 Solute and matric stress-affected gene functions identified by plasposon mutagenesis in *Novosphingobium* sp. LH128

Mutant strain	Closest identified protein	Organism	% identity/ % similarity	E value	LH128 protein accession no.[a]	Protein family (Pfam accession no.)
PE8G11	Hypothetical protein	*Idiomarina loihiensis* L2TR	45/59	$9e^{-36}$	EJU14233	Unknown function (DUF2092)
PE11E1	Major facilitator superfamily transporter	*Rhodospirillum rubrum* ATCC 11170	36/48	$6e^{-25}$	EJU13375	Major facilitator family (PF07690)
PE11G11	23S rRNA	*Novosphingobium* sp. strain PP1Y	96[b]	0		
PE15E1	Flagellar hook length control protein FliK	*Novosphingobium* sp. strain AP12	46/58	$8e^{-29}$	EJU10691	Flagellar hook length control protein FliK (PF02120)
PE26E12	Fe^{2+}/Zn^{2+} uptake regulator, Fur family	*Novosphingobium* sp. AP12	94/96	$4e^{-93}$	EJU12308	Ferric uptake regulator family (PF01475)
PE37C12	Putative promoter region of hypothetical protein	*Sphingomonas* sp. strain PAMC 26621	52/65	$7e^{-72}$	EJU13325	No Pfam match
PE37E8	Putative outer membrane protein	*Novosphingobium nitrogenifigens* DSM 19370	31/48	$4e^{-52}$	EJU12654	Member of clan CL0193 (outer membrane beta-barrel protein superfamily) (DUF2320)
PE38B5	Malate dehydrogenase	*Novosphingobium* sp. strain Rr 2-17	87/93	0	EJU10524	Malic enzyme, N-terminal domain (PF0039)
PE38G5	Unknown function				EJU11217	No Pfam match
PE43E10	Outer membrane protein (TonB)-dependent receptor	*Novosphingobium* sp. AP12	86/92	0	EJU09816	TonB-dependent receptor (PF00593)
PE44F8	5′-Nucleotidase	*Novosphingobium* sp. Rr 2-17	80/88	$2e^{-06}$	EJU13330	5′-Nucleotidase, C terminal (PF02872)
PE49E6	Aldose 1-epimerase	*Novosphingobium* sp. AP12	90/94	0	EJU10999	Aldose 1-epimerase (PF01263)
PE49E7	Diguanylate cyclase/ phosphodiesterase (GGDEF)	*Novosphingobium* sp. AP12	81/87	0	EJU09483	GGDEF (PF00990) and PAS domain (PF00989)-containing protein
PE51B4	AAA ATPase	*Burkholderia glumae* BGR1	59/69	$7e^{-07}$	EJU15112	AAA ATPase domain (PF00004)
NA7E1	Outer membrane protein of *Sphingomonas* (OpsA)	*Novosphingobium* sp. PP1Y	78/82	$4e^{-145}$	EJU13611	Outer membrane protein A (PF00691)
NA8B8	Nucleotide-binding protein	*Novosphingobium* sp. PP1Y	52/71	$9e^{-126}$	EJU14827	CobQ/CobB/MinD/ParA nucleotide-binding domain (PF01656)
NA36A2	Putative DEAD/DEAH box helicase-like protein	*Bradyrhizobium* sp. strain WSM1253	68/78	0	EJU11915	Helicase conserved C-terminal domain (PF00271)
NA36D9	Outer membrane protein (TonB)-dependent receptor	*Novosphingobium* sp. PP1Y	70/81	0	EJU11595	TonB-dependent receptor (PF00593)
NA40B6	Unknown function					
NA43C6	Na^+/solute symporter	*Sphingomonas sanxanigenens* DSM16654	86/92	0	EJU10925	No Pfam match
NA44B7	Hypothetical protein	*Burkholderia ambifaria* AMMD	50/65	$4e^{-51}$	EJU13777	No Pfam match
NA45C10	Hypothetical protein	*Sphingobium indicum* B90A	41/58	0.001	EJU11509	No Pfam match
NA45D6	Putative phosphotransferase family protein	*Sphingobium chlorophenolicum* L-1	88/93	0	EJU09392	Phosphotransferase enzyme family (PF01636)
NA46E5	Glucan biosynthesis protein G	*Novosphingobium* sp. PP1Y	67/76	0	EJU12821	Periplasmic glucan biosynthesis protein MdoG (PF04349)
NA46G6	NADPH-dependent flavin mononucleotide reductase	*Novosphingobium* sp. PP1Y	70/80	$6e^{-79}$	EJU14890	NADPH-dependent flavin mononucleotide reductase (PF03358)

[a] Protein nominations are provided in the draft genome sequence project of LH128, deposited at DDBJ/EMBL/GenBank under accession number ALVC00000000; the accession number of the first version is ALVC01000000.
[b] The value is based on nucleotide sequence identity.

threne degradation (Fig. 6A). Whereas about 90% of the added phenanthrene was degraded in TM soil inoculated with wild-type strain LH128, only 60% was degraded in the NA7E1-inoculated TM soil. Also, at 25% of the WHC, the wild type survived better than NA7E1, whose population size declined to below the detection limit after 10 days of incubation (Fig. 5A). However, at 25% of the WHC in sterile TM soil, the degradation of the added phenanthrene was insignificant (Fig. 6A). Similarly, in nonsterile TM soil at the WHC, the wild type maintained its population size, whereas that of NA7E1 decreased to below the detection limit after 10 days of incubation (Fig. 5B). In this case, about 85% phenanthrene was degraded in the microcosms inoculated with the wild type, but none was degraded in those inoculated with NA7E1 (Fig. 6B). As in sterile TM soil, no survival was observed for NA7E1 inoculated in nonsterile TM soil at 25% of the WHC, whereas the wild type survived clearly better. However, no significant degradation of phenanthrene was observed even in the case of the wild type (Fig. 5B and Fig. 6B). In a separate experiment, the survival of *opsA*-complemented mutant strain NA7E1 in sterile TM soil at 25% of the WHC was compared with that of wild-type strain LH28 and mutant NA7E1. The survival of the complemented mutant NA7E1 in soil was similar to that of the wild-type strain (Fig. 7), while the survival of mutant NA7E1 was severely affected.

The survival of mutant NA7E1 compared to that of the wild type was additionally tested in a historically PAH-contaminated soil (Sobeslav soil) additionally amended with phenanthrene. In all cases, as observed in the TM soil, a rapid decline in the numbers of CFU was observed 4 h after inoculation for both wild-type

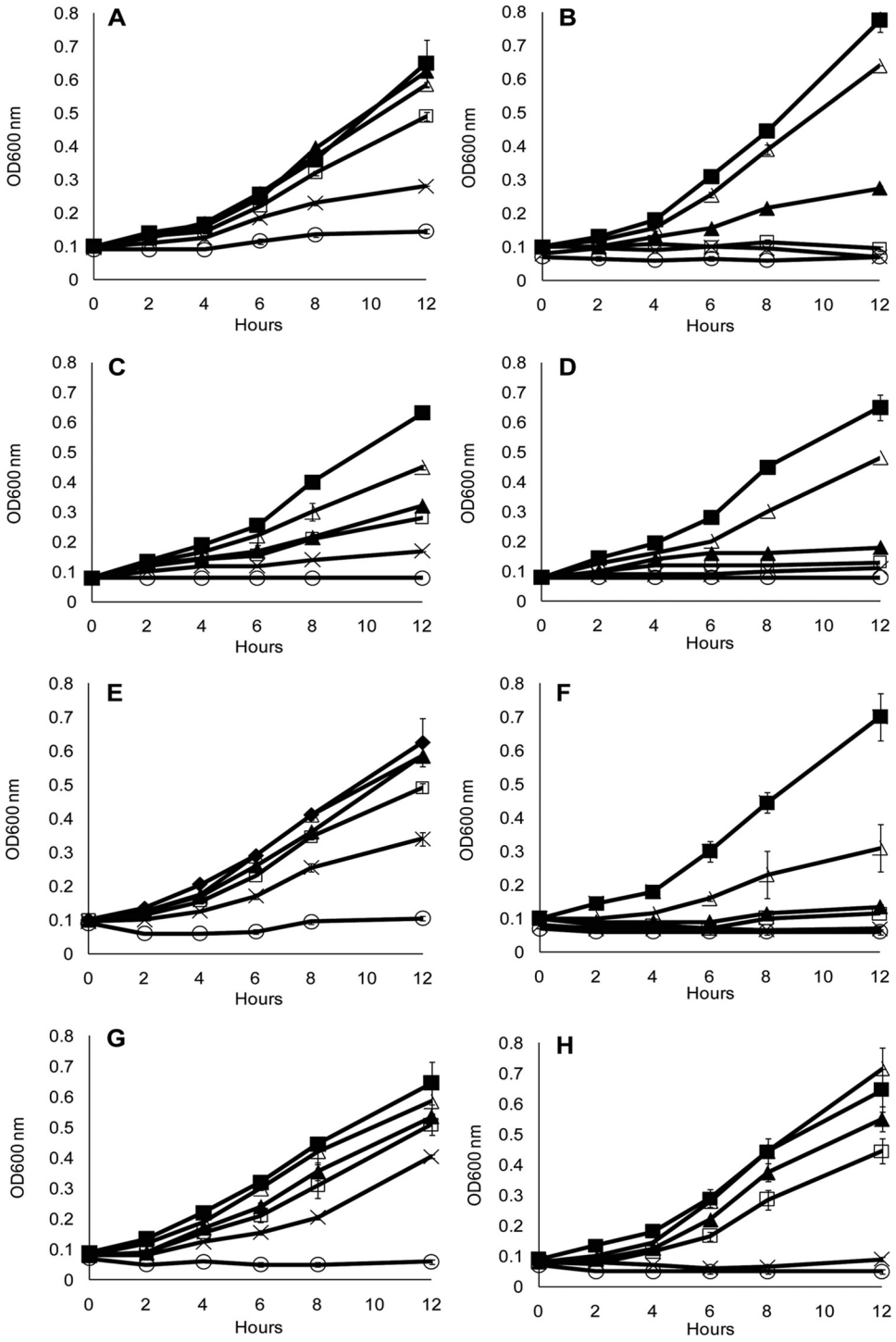

FIG 1 Effect of increasing concentrations of solutes on growth on glucose of wild-type strain *Novosphingobium* sp. LH128 and *opsA* mutant NA7E1. (A) Wild-type LH128/NaCl; (B) NA7E1/NaCl; (C) wild-type LH128/LiCl; (D) NA7E1/LiCl; (E) wild-type LH128/Na_2SO_4; (F) NA7E1/Na_2SO_4; wild-type LH128/sucrose (G); NA7E1/sucrose (H). Symbols: ■, control; Δ, 100 mM salt; ▲, 200 mM salt; □, 300 mM salt; ×, 400 mM salt; and ○, 600 mM salt. The OD_{600} values represent the averages derived from 3 replicate cultures. Where the standard deviations are not visible, they are hidden behind the symbols.

strain LH128 and NA7E1 independently of the soil moisture content and the sterility of the soil (Fig. 5C and D). In sterile Sobeslav soil at the WHC, wild-type strain LH128 survived over a period of 20 days, whereas the numbers for NA7E1 dropped below the detection limit after 10 days (Fig. 5C). About 50% of the added phenanthrene was degraded in soil inoculated with wild-type strain LH128, but only 15% of the added phenanthrene was degraded in the case of NA7E1 (Fig. 6C). In sterile Sobeslav soil at 25% of the WHC, the numbers of CFU of both NA7E1 and the wild type declined rapidly immediately after inoculation (to 1.0 ×

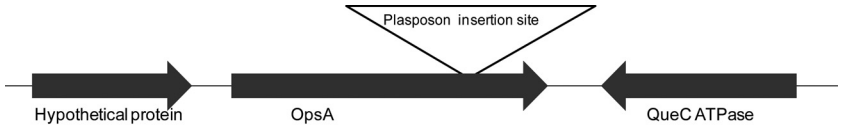

FIG 2 Genetic context of *opsA* in *Novosphingobium* sp. LH128 showing the location of the plasposon insertion in mutant NA7E1. The direction of transcription of the respective ORFs is indicated by the arrow. QueC ATPase, queuosine biosynthesis QueC ATPase.

10^3 and 1.67×10^4 CFU g^{-1}, respectively), and neither of the strains was recovered after 10 days of incubation (Fig. 5C). Still, about 30% of the added phenanthrene was degraded in the case of inoculation with the wild type, but none of the added phenanthrene was degraded in the case of inoculation with NA7E1 (Fig. 6C). In nonsterile soil at the WHC, both the wild-type and NA7E1 populations decreased to below the detection limit after 10 days of incubation (Fig. 5D). However, about 40% of the added phenanthrene was still degraded in both systems (Fig. 6D). In contrast, in microcosms containing nonsterile Sobeslav soil with a moisture content of 25% of the WHC, the wild type still survived at day 10 with a concomitant degradation of about 50% of the phenanthrene. The NA7E1 population size in this case again decreased to below the detection limit without apparent phenanthrene degradation (Fig. 5D and 6D).

Finally, survival and activity experiments were performed with mutant NA7E1 in phenanthrene-amended sterile sand that was washed or not washed with Milli-Q water and that had a moisture content of its WHC or 25% of its WHC. The sand was washed to remove solutes and, as such, to decrease the solute concentration in the pore water. In nonwashed sand, the final number of CFU of the mutant strain decreased significantly more than that of wild-type strain LH128, regardless of the moisture content ($P < 0.001$). In contrast, in washed sand, both populations developed equally (Fig. 5E and F). However, neither population growth nor a significant improvement in phenanthrene degradation was observed for both inocula under both moisture conditions and irrespective of sand washing (Fig. 6E and F).

DISCUSSION

A genome-wide transposon mutagenesis approach was used to identify genes important for survival under solute and matric stress conditions in *Novosphingobium* sp. LH128 using a Tn*5*-based plasposon system. The majority of the identified matric or solute stress-related genes have not previously been reported to be associated with those stress conditions. However, some of the affected genes have been associated with a general stress response. Some of the disrupted putative gene functions in mutants prone to matric stress could be linked to transport functions. For instance, insertions occurred in genes whose deduced protein sequence showed homology to a major facilitator superfamily (MFS) transporter (mutant PE11E1), the TonB-dependent receptor (mutant PE43E10), and a putative outer membrane protein (mutant PE37E8). The plasposon insertion in mutant PE26E12 is in a gene whose putative protein product shares homology with the FurB Fe^{2+}/Zn^{2+} uptake regulator that has been related to the uptake of zinc but also to the oxidative stress response in *Mycobacterium tuberculosis* (45). The gene affected in mutant PE49E7 encodes a homologue of diguanylate cyclase/phosphodiesterase, which regulates bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), a bacterial second messenger that plays a role in many physiological processes, including motility, cell differentiation, biofilm formation, virulence, cell-to-cell signaling, and the general stress response in bacteria such as *E. coli* and *Legionella pneumophila* (46, 47). Differential expression of aldose 1-epimerase, the putative product of the gene affected in mutant PE49E6, has also been shown in *E. coli* during temperature stress (48). The putative aldose 1-epimerase gene in LH128 does not seem to have further downstream genes within the same operon; therefore, polar effects are unlikely to explain the phenotype of mutant PE49E6.

Unlike the mutants showing increased sensitivity to matric stress, many of the mutants prone to solute stress were affected in genes encoding putative proteins which could be directly linked with solute stress mitigation. These include membrane proteins, such as a putative outer membrane protein in mutant NA7E1 (but in an ORF different from that affected in mutant PE37E8), a putative TonB-dependent receptor in mutant NA36D9 (but in an ORF different from that affected in mutant PE43E10), and a putative glucan biosynthesis protein G (NA46E5). The gene affected

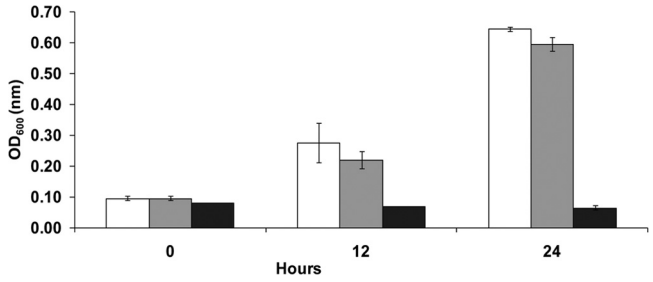

FIG 3 Effect of NaCl (450 mM) on growth on glucose of wild-type strain *Novosphingobium* sp. LH128 (white bars), complemented *opsA* mutant NA7E1 (gray bars), and noncomplemented *opsA* mutant NA7E1 (black bars).

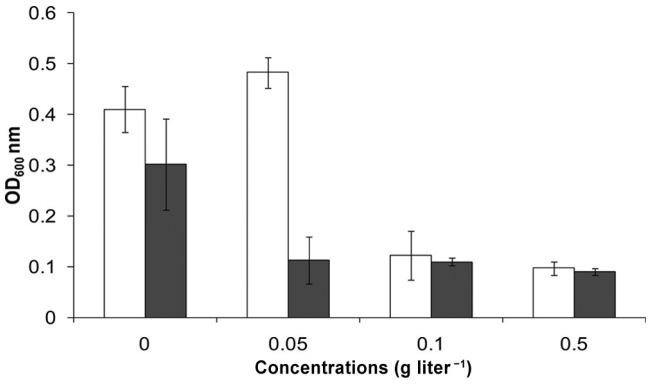

FIG 4 Effect of increasing concentrations of SDS on growth of wild-type strain *Novosphingobium* sp. LH128 (white bars) and *opsA* mutant NA7E1 on glucose (gray bars). The OD_{600} values represent the average values derived from 3 replicate samples after 16 h of incubation. The standard deviations are indicated.

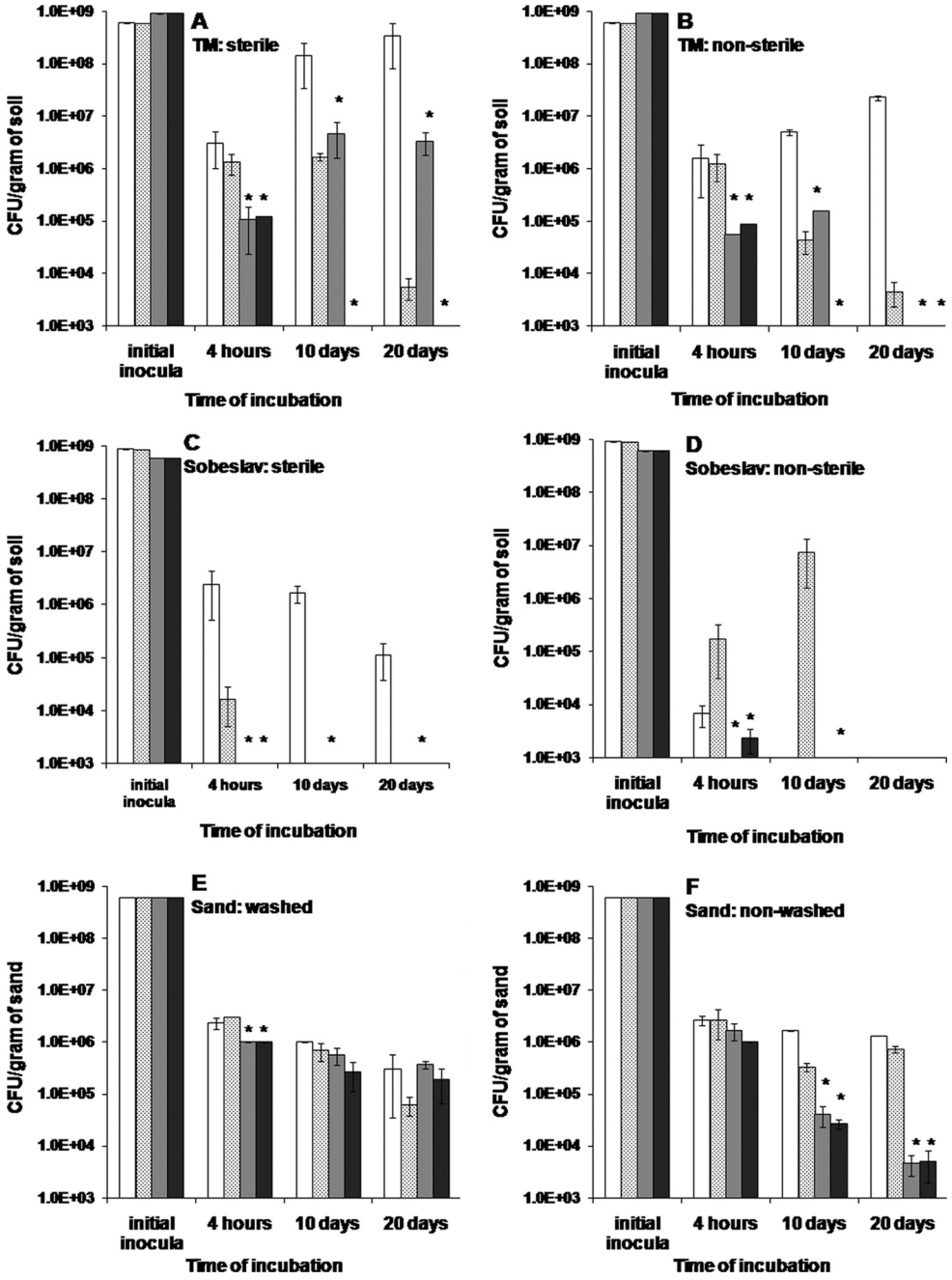

FIG 5 Survival of *Novosphingobium* sp. LH128 and *opsA* mutant NA7E1 in soil with a moisture content at the WHC or 25% of the WHC supplemented with 500 mg kg^{-1} phenanthrene. (A) Sterile TM soil; (B) nonsterile TM soil; (C) sterile Sobeslav soil; (D) nonsterile Sobeslav soil; (E) washed sand; (F) nonwashed sand. White bars, wild-type strain LH128 at the WHC; dotted bars, wild-type strain LH128 at 25% of the WHC; gray bars, NA7E1 at the WHC; black bars, NA7E1 at 25% of the WHC. The numbers of CFU shown represent the average values derived from 3 replicate microcosms. The standard deviations are indicated. In case no average value is given, the value was below the detection limit of 1×10^3 CFU per gram of soil. *, significant difference between wild-type strain LH128 and NA7EA at the respective moisture contents and time points.

in mutant NA36D9 encodes a homologue of a TonB-dependent receptor, an outer membrane protein that interacts with the cytoplasmic membrane protein TonB to mediate the uptake of solutes such as iron-siderophore complexes, carbohydrates, and vitamin B_{12}, for instance, in *E. coli* (49, 50). To our knowledge, there is no known osmoregulatory gene that is TonB dependent. The gene identified in mutant NA46E5 encodes a homologue of the glucan biosynthesis protein G that is required for the synthesis of periplasmic glucans, a family of oligosaccharides in the periplasmic space of Gram-negative bacteria that are important for the stability of the membrane (51, 52). Increased expression of a gene encoding the glucan biosynthesis protein G was also reported in LH128 biofilms and *Sphingomonas wittichii* RW1 affected by solute stress (30, 31). Finally, in mutant NA7E1, a gene designated

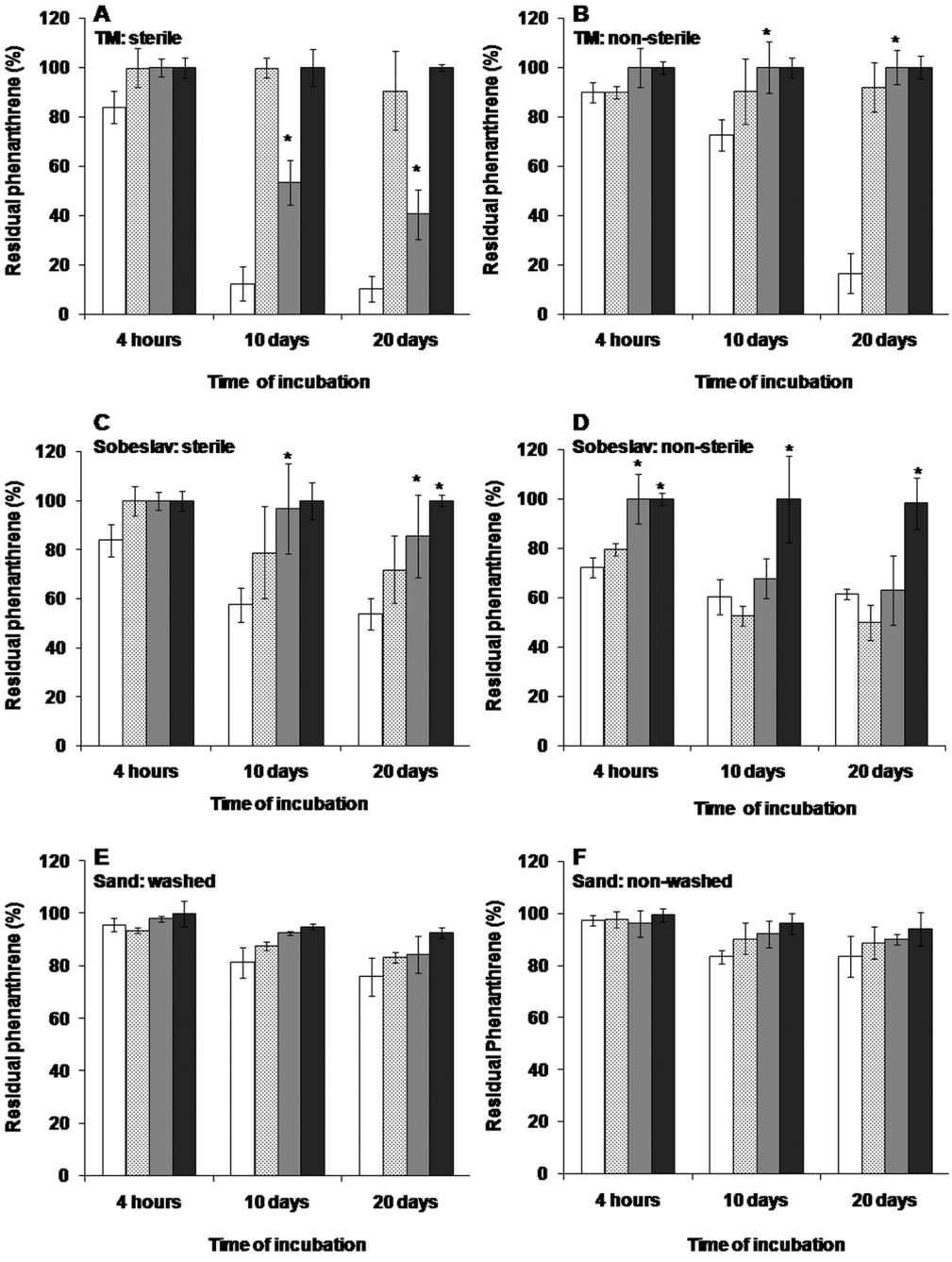

FIG 6 Phenanthrene degradation by *Novosphingobium* sp. LH128 and *opsA* mutant NA7E1 in soil with a moisture content at the WHC or 25% of the WHC supplemented with 500 mg kg^{-1} phenanthrene. The residual amounts of phenanthrene (presented as a percentage of the initial phenanthrene added) in sterile TM soil (A), nonsterile TM soil (B), sterile Sobeslav soil (C), nonsterile Sobeslav soil (D), washed sand (E), and nonwashed sand (F) are shown. White bars, wild-type strain LH128 at the WHC; dotted bars, wild-type strain LH128 at 25% of the WHC; gray bars, NA7E1 at the WHC; black bars, NA7E1 at 25% of the WHC. The values represent the average values derived from 3 replicate microcosms. The standard deviations are indicated. *, significant difference between wild-type strain LH128 and NA7EA at the respective moisture contents and time points.

opsA encoding a putative outer membrane protein was affected. This gene is also present in other sphingomonad species, such as *Sphingomonas wittichii* RW1 (Swit_2278), but is annotated as OmpA/MotB and showed increased expression during solute stress in RW1 (31). The association with MotB is due to the high similarity of the peptidoglycan-binding C-terminal domain of OmpA-like proteins with MotB (53) but does not imply that the protein is implicated in flagellar motility like MotB is. This was confirmed by motility tests in soft agar or microscopic observations, which verified that the motility of mutant NA7E1 was not affected (data not shown). The corresponding protein contains 337 amino acid residues, including a Sec-dependent secretion signal peptide (21 amino acids) that is cleaved off upon secretion. The protein has two domains separated by a proline-rich stretch

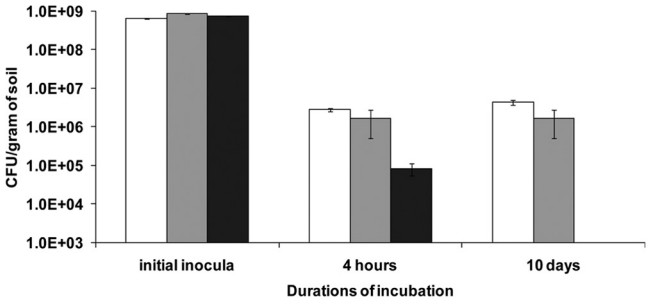

FIG 7 Survival in sterile TM soil at 25% of the WHC of wild-type strain *Novosphingobium* sp. LH128 (white bars), complemented *opsA* mutant NA7E1 (gray bars), and noncomplemented *opsA* mutant NA7E1 (black bars). The values represent the average values derived from 3 replicate microcosms. The standard deviations are indicated.

and shows homology with the well-characterized OmpA protein in *E. coli* K-12 in the C-terminal peptidoglycan-binding part (41% amino acid identity), while it lacks similarity in the N-terminal domain. OmpA in *E. coli* has been shown to be involved in osmoprotection (54, 55) but also functions as a bacteriophage receptor (56) and has been shown to be involved in F-factor-dependent conjugation (57) and in biofilm formation (58). Moreover, the C-terminal domains have been suggested to be anchored with peptidoglycan in both Gram-positive and Gram-negative bacteria (53). The growth of mutant NA7E1 was impaired not only in the presence of NaCl but also in the presence of other ionic solutes (from about 200 mM) and sucrose at 400 mM, while it was not affected by PEG 6000, which, rather, reduced water activity by macromolecule-water interactions (59, 60). These observations suggest that OpsA in strain LH128 is important for alleviating not only NaCl stress but also stress from other solutes. The different solutes impacted the growth of the mutant, though they did so at nonequivalent decreases of water potential. NaCl and LiCl impacted growth at a decrease of water potential of 0.74 MPa, while Na_2SO_4 did so at a decrease of 1.1 MPa. This can be explained by the additional effects of the chlorine ions in the case of NaCl and LiCl (61). PEG 6000, as a nonpermeating substance, did not impair the growth of the mutant at the concentrations used, but the highest concentration used of 10% resulted in a reduction in water potential equivalent to 0.18 MPa, which is lower than the reductions that occurred in the case of the solutes. Higher PEG 6000 concentrations were not tested, since the wild-type strain was already affected at 10% PEG 6000. Furthermore, the sensitivity of mutant NA7E1 to SDS detergent suggests that OpsA might have a structural role in the membrane and is involved in membrane stability, as has also been suggested for OmpA in *E. coli* (55). Hence, the effect of the increased solute concentrations in the *opsA* mutant might also be explained by a decrease in membrane stability.

Most of the tested mutants identified as disturbed in matric or solute stress resistance on agar plates did not show impaired survival in soil even in the presence of a reduced moisture content and, hence, behaved like the wild-type strain. This implies that the genes identified to be important for coping with matric or solute stress in these mutants under laboratory conditions may not be relevant in soil or that the stress levels in the soil systems used were not sufficiently high to affect survival and activity. Other data obtained with *S. wittichii* RW1 suggest that interruption of numerous different (nonessential) genes caused a loss of fitness for growth in soil (31). Our preliminary data showed that the drastic decline in the numbers of CFU of the wild-type strain shortly after inoculation was due to the entrance of cells into a viable-but-nonculturable (VBNC)-like state and was not due to cell death (data not shown). The decline in the numbers of CFU was not observed in cells incubated in $MgSO_4$ for both the wild type and the mutants (data not shown). The decline in the numbers of CFU was also not related to the release of toxic substances due to soil autoclaving, since the same effect was observed in nonsterile soils. The only mutant that behaved differently from the wild-type strain was mutant NA7E1 carrying the *opsA* mutation, which was affected in growth on glucose at much lower NaCl concentrations than the other mutants. Both the growth and the phenanthrene degradation activity of mutant NA7E1 were severely impaired in sterile and nonsterile soils compared to those of the wild type. We hypothesize that this is related to the decreased water potential and increased solute stress in soil, with which the *opsA* mutant can cope less easily than the wild-type strain because of its decreased ability to mitigate solute stress or decreased membrane stability. This hypothesis is supported by the observation that the survival of the mutant in soil with decreased moisture content was significantly poorer than that of the wild type and was higher in washed than in nontreated sand. We also noticed that the survival of mutant NA7E1 upon extended incubation on desiccating agarose plates declined more rapidly than that of the wild-type strain. The observation that, even in soil with a moisture content at the WHC, the *opsA* mutation reduced survival compared to that of the wild type suggests that *opsA* is important for the survival of LH128 in soil. Other studies have identified genes important for soil colonization in organisms like *Pseudomonas fluorescens* (62) and *Burkholderia multivorans* (63) using *in vivo* expression technology, but those gene functions were not related to soil stress situations like moisture stress. The observed reduction in growth was more severe in nonsterile than sterile soil for both TM soil and the historically PAH-contaminated Sobeslav soil, but this was also the case for wild-type strain LH128. This was not unexpected, since biotic stress due to competition for space or nutrients or to predation can largely affect the survival and growth of foreign bacteria introduced into soil and is considered an important factor in determining the success of bioaugmentation for bioremediation purposes (64). Due to the *opsA* deficiency, mutant NA7E1 clearly lost its competitive ability to survive in the presence of other microorganisms. Interestingly, wild-type strain LH128 survived better in the historically PAH-contaminated nonsterile Sobeslav soil when the moisture content was at 25% of the WHC than when the moisture content corresponded to its WHC. The decline in the numbers of CFU after soil inoculation was likely not due to toxic compounds released during soil sterilization, as the wild-type strain survived better in saturated sterile soil than nonsterile TM and Sobeslav soils. Moreover, while phenanthrene degradation in the nonsterile Sobeslav soil at the WHC was probably partly due to activity by the endogenous community, in the case of a moisture content of 25% of the WHC, it was apparently due to LH128 activity, since degradation was not observed in the corresponding microcosms inoculated with the *opsA* mutant. Furthermore, the survival rate and PAH biodegradation activity of both wild-type strain LH128 and the mutant depended on the tested soil, which can be linked to the different soil compositions and characteristics. For instance, wild-type strain LH128 survived and performed less well in the Sobeslav

soil than the TM soil. This could be due to the presence of toxic compounds in the Sobeslav soil. In the sand microcosms, no improvement in degradation or growth was observed, despite strain survival. In this case, the failure could be due to the absence of nutrients or the presence of a very low concentration of nutrients essential for growth and phenanthrene degradation in the sand substrate used.

In conclusion, this study identifies gene functions in PAH-degrading soil sphingomonads which are important for them to cope with matric and solute stress. Moreover, it identifies that *opsA* is important for the survival of *Novosphingobium* sp. LH128 in soil and for the strain to deal with osmotic and moisture stress.

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REFERENCES

1. **Fiala Z, Vyskocil A, Krajak V, Masin V, Emminger S, Srb V, Tejral J.** 1999. Polycyclic aromatic hydrocarbons. I. Environmental contamination and environmental exposure. Acta Medica (Hradec Kralove) Suppl. **42:** 77–89. (In Czech.)
2. **Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, Diels L.** 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl. Environ. Microbiol. **66:**1834–1843. http://dx.doi.org/10.1128/AEM.66.5.1834-1843.2000.
3. **Leys NM, Ryngaert A, Bastiaens L, Verstraete W, Top EM, Springael D.** 2004. Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **70:**1944–1955. http://dx.doi.org/10.1128/AEM.70.4.1944-1955.2004.
4. **Jones MD, Singleton DR, Sun W, Aitken MD.** 2011. Multiple DNA extractions coupled with stable-isotope probing of anthracene-degrading bacteria in contaminated soil. Appl. Environ. Microbiol. **77:**2984–2991. http://dx.doi.org/10.1128/AEM.01942-10.
5. **Johnsen AR, Winding A, Karlson U, Roslev P.** 2002. Linking of microorganisms to phenanthrene metabolism in soil by analysis of ^{13}C-labeled cell lipids. Appl. Environ. Microbiol. **68:**6106–6113. http://dx.doi.org/10.1128/AEM.68.12.6106-6113.2002.
6. **Cai M, Xun LY.** 2002. Organization and regulation of pentachlorophenol-degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. J. Bacteriol. **184:**4672–4680. http://dx.doi.org/10.1128/JB.184.17.4672-4680.2002.
7. **Johannesen H, Sorensen SR, Aamand J.** 2003. Mineralization of soil-aged isoproturon and isoproturon metabolites by *Sphingomonas* sp. strain SRS2. J. Environ. Qual. **32:**1250–1257. http://dx.doi.org/10.2134/jeq2003.1250.
8. **Wittich R-M, Wilkes H, Sinnwell V, Francke W, Fortnagel P.** 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl. Environ. Microbiol. **58:**1005–1010.
9. **Nohynek LJ, Suhonen EL, Nurmiaho-Lassila E-L, Hantula J, Salkinoja-Salonen M.** 1995. Description of four pentachlorophenol-degrading bacterial strains as *Sphingomonas chlorophenolica* sp. nov. Syst. Appl. Microbiol. **18:**527–538. http://dx.doi.org/10.1016/S0723-2020(11)80413-3.
10. **Vorob'eva LI.** 2004. Stressors, stress reactions, and survival of bacteria (a review). Appl. Biochem. Microbiol. **40:**261–269. (In Russian.) http://dx.doi.org/10.1023/B:ABIM.0000025941.11643.19.
11. **Kultz D.** 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J. Exp. Biol. **206:**3119–3124. http://dx.doi.org/10.1242/jeb.00549.
12. **Ramos JL, Gallegos MT, Marques S, Ramos-Gonzalez MI, Espinosa-Urgel M, Segura A.** 2001. Responses of Gram-negative bacteria to certain environmental stressors. Curr. Opin. Microbiol. **4:**166–171. http://dx.doi.org/10.1016/S1369-5274(00)00183-1.
13. **Goldstein RM, Mallory LM, Alexander M.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. **50:**977–983.
14. **Holden PA, Halverson LJ, Firestone MK.** 1997. Water stress effects on toluene biodegradation by *Pseudomonas putida*. Biodegradation **8:**143–151. http://dx.doi.org/10.1023/A:1008237819089.
15. **Shabala L, Bowman J, Brown J, Ross T, McMeekin T, Shabala S.** 2009. Ion transport and osmotic adjustment in *Escherichia coli* in response to ionic and non-ionic osmotica. Environ. Microbiol. **11:**137–148. http://dx.doi.org/10.1111/j.1462-2920.2008.01748.x.
16. **Chang WS, Li X, Halverson LJ.** 2009. Influence of water limitation on endogenous oxidative stress and cell death within unsaturated *Pseudomonas putida* biofilms. Environ. Microbiol. **11:**1482–1492. http://dx.doi.org/10.1111/j.1462-2920.2009.01876.x.
17. **Mantell A, Frenkel H, Meiri A.** 1985. Drip irrigation of cotton with saline-sodic water. Irrig. Sci. **6:**95–106. http://dx.doi.org/10.1007/BF00251558.
18. **Ramakrishna DM, Viraraghavan T.** 2005. Environmental impact of chemical deicers—a review. Water Air Soil Pollut. **166:**49–63. http://dx.doi.org/10.1007/s11270-005-8265-9.
19. **Johnsen AR, de Lipthay JR, Sorensen SJ, Ekelund F, Christensen P, Andersen O, Karlson U, Jacobsen CS.** 2006. Microbial degradation of street dust polycyclic aromatic hydrocarbons in microcosms simulating diffuse pollution of urban soil. Environ. Microbiol. **8:**535–545. http://dx.doi.org/10.1111/j.1462-2920.2005.00935.x.
20. **Poolman B, Blount P, Folgering JH, Friesen RH, Moe PC, van der Heide T.** 2002. How do membrane proteins sense water stress? Mol. Microbiol. **44:**889–902. http://dx.doi.org/10.1046/j.1365-2958.2002.02894.x.
21. **Van de Mortel M, Halverson LJ.** 2004. Cell envelope components contributing to biofilm growth and survival of *Pseudomonas putida* in low-water-content habitats. Mol. Microbiol. **52:**735–750. http://dx.doi.org/10.1111/j.1365-2958.2004.04008.x.
22. **Rhodius VA, Suh WC, Nonaka G, West J, Gross CA.** 2006. Conserved and variable functions of the σ^E stress response in related genomes. PLoS Biol. **4:**e2. http://dx.doi.org/10.1371/journal.pbio.0040002.
23. **Boor KJ.** 2006. Bacterial stress responses: what doesn't kill them can make them stronger. PLoS Biol. **4:**e23. http://dx.doi.org/10.1371/journal.pbio.0040023.
24. **Record MT, Jr, Courtenay ES, Cayley DS, Guttman HJ.** 1998. Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. Trends Biochem. Sci. **23:**143–148.
25. **Barth S, Huhn M, Matthey B, Klimka A, Galinski EA, Engert A.** 2000. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl. Environ. Microbiol. **66:**1572–1579. http://dx.doi.org/10.1128/AEM.66.4.1572-1579.2000.
26. **Gunasekera TS, Csonka LN, Paliy O.** 2008. Genome-wide transcriptional responses of *Escherichia coli* K-12 to continuous osmotic and heat stresses. J. Bacteriol. **190:**3712–3720. http://dx.doi.org/10.1128/JB.01990-07.
27. **Aspedon A, Palmer K, Whiteley M.** 2006. Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. J. Bacteriol. **188:**2721–2725. http://dx.doi.org/10.1128/JB.188.7.2721-2725.2006.
28. **Hazen TC, Stahl DA.** 2006. Using the stress response to monitor process control: pathways to more effective bioremediation. Curr. Opin. Biotechnol. **17:**285–290. http://dx.doi.org/10.1016/j.copbio.2006.03.004.
29. **Johnson D, Coronado E, Moreno-Forero S, Heipieper H, van der Meer J.** 2011. Transcriptome and membrane fatty acid analyses reveal different strategies for responding to permeating and non-permeating solutes in the bacterium *Sphingomonas wittichii*. BMC Microbiol. **11:**250. http://dx.doi.org/10.1186/1471-2180-11-250.
30. **Fida TT, Breugelmans P, Lavigne R, Coronado E, Johnson DR, van der Meer JR, Mayer AP, Heipieper HJ, Hofkens J, Springael D.** 2012. Exposure to solute stress affects genome-wide expression but not the polycyclic aromatic hydrocarbon-degrading activity of *Sphingomonas* sp. strain LH128 in biofilms. Appl. Environ. Microbiol. **78:**8311–8320. http://dx.doi.org/10.1128/AEM.02516-12.
31. **Roggo C, Coronado E, Moreno-Forero SK, Harshman K, Weber J, van der Meer JR.** 2013. Genome-wide transposon insertion scanning of environmental survival functions in the polycyclic aromatic hydrocarbon degrading bacterium *Sphingomonas wittichii* RW1. Environ. Microbiol. **15:**2681–2695. http://dx.doi.org/10.1111/1462-2920.12125.
32. **Busse MD, Bottomley PJ.** 1989. Growth and nodulation responses of *Rhizobium meliloti* to water stress induced by permeating and nonpermeating solutes. Appl. Environ. Microbiol. **55:**2431–2436.
33. **Csonka LN.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.

34. **Csonka LN, Ikeda TP, Fletcher SA, Kustu S.** 1994. The accumulation of glutamate is necessary for optimal growth of *Salmonella typhimurium* in media of high osmolality but not induction of the *proU* operon. J. Bacteriol. **176:**6324–6333.
35. **Halverson LJ, Firestone MK.** 2000. Differential effects of permeating and nonpermeating solutes on the fatty acid composition of *Pseudomonas putida*. Appl. Environ. Microbiol. **66:**2414–2421. http://dx.doi.org/10.1128/AEM.66.6.2414-2421.2000.
36. **Aylward FO, McDonald BR, Adams SM, Valenzuela A, Schmidt RA, Goodwin LA, Woyke T, Currie CR, Suen G, Poulsen M.** 2013. Comparison of 26 sphingomonad genomes reveals diverse environmental adaptations and biodegradative capabilities. Appl. Environ. Microbiol. **79:**3724–3733. http://dx.doi.org/10.1128/AEM.00518-13.
37. **Fida TT, Moreno-Forero SK, Heipieper HJ, Springael D.** 2013. Physiology and transcriptome of the polycyclic aromatic hydrocarbon-degrading *Sphingomonas* sp. LH128 after long-term starvation. Microbiology **159:**1807–1817. http://dx.doi.org/10.1099/mic.0.065870-0.
38. **Reasoner DJ, Geldreich EE.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:**1–7.
39. **Uyttebroek M, Ortega-Calvo JJ, Breugelmans P, Springael D.** 2006. Comparison of mineralization of solid-sorbed phenanthrene by polycyclic aromatic hydrocarbon (PAH)-degrading *Mycobacterium* spp. and *Sphingomonas* spp. Appl. Microbiol. Biotechnol. **72:**829–836. http://dx.doi.org/10.1007/s00253-006-0337-2.
40. **Larsen R, Wilson M, Guss A, Metcalf W.** 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch. Microbiol. **178:**193–201. http://dx.doi.org/10.1007/s00203-002-0442-2.
41. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
42. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. **Michel BE.** 1983. Evaluation of the water potentials of solutions of polyethylene glycol 8000 both in the absence and presence of other solutes. Plant Physiol. **72:**66–70. http://dx.doi.org/10.1104/pp.72.1.66.
44. **Breugelmans P, D'Huys PJ, De Mot R, Springael D.** 2007. Characterization of novel linuron-mineralizing bacterial consortia enriched from long-term linuron-treated agricultural soils. FEMS Microbiol. Ecol. **62:**374–385. http://dx.doi.org/10.1111/j.1574-6941.2007.00391.x.
45. **Lucarelli D, Vasil ML, Meyer-Klaucke W, Pohl E.** 2008. The metal-dependent regulators FurA and FurB from *Mycobacterium tuberculosis*. Int. J. Mol. Sci. **9:**1548–1560. http://dx.doi.org/10.3390/ijms9081548.
46. **Levet-Paulo M, Lazzaroni JC, Gilbert C, Atlan D, Doublet P, Vianney A.** 2011. The atypical two-component sensor kinase Lpl0330 from *Legionella pneumophila* controls the bifunctional diguanylate cyclase-phosphodiesterase Lpl0329 to modulate bis-(3′-5′)-cyclic dimeric GMP synthesis. J. Biol. Chem. **286:**31136–31144. http://dx.doi.org/10.1074/jbc.M111.231340.
47. **Weber H, Pesavento C, Possling A, Tischendorf G, Hengge R.** 2006. Cyclic-di-GMP-mediated signalling within the σ^S network of *Escherichia coli*. Mol. Microbiol. **62:**1014–1034. http://dx.doi.org/10.1111/j.1365-2958.2006.05440.x.
48. **Jones TH, Murray A, Johns M, Gill CO, McMullen LM.** 2006. Differential expression of proteins in cold-adapted log-phase cultures of *Escherichia coli* incubated at 8, 6 or 2°C. Int. J. Food Microbiol. **107:**12–19. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.08.006.
49. **Ferguson AD, Deisenhofer J.** 2002. TonB-dependent receptors—structural perspectives. Biochim. Biophys. Acta **1565:**318–332. http://dx.doi.org/10.1016/S0005-2736(02)00578-3.
50. **Noinaj N, Guillier M, Barnard TJ, Buchanan SK.** 2010. TonB-dependent transporters: regulation, structure, and function. Annu. Rev. Microbiol. **64:**43–60. http://dx.doi.org/10.1146/annurev.micro.112408.134247.
51. **Page F, Altabe S, Hugouvieux-Cotte-Pattat N, Lacroix J-M, Robert-Baudouy J, Bohin J-P.** 2001. Osmoregulated periplasmic glucan synthesis is required for *Erwinia chrysanthemi* pathogenicity. J. Bacteriol. **183:**3134–3141. http://dx.doi.org/10.1128/JB.183.10.3134-3141.2001.
52. **Bohin JP.** 2000. Osmoregulated periplasmic glucans in proteobacteria. FEMS Microbiol. Lett. **186:**11–19. http://dx.doi.org/10.1016/S0378-1097(00)00110-5.
53. **De Mot R, Vanderleyden J.** 1994. The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both Gram-positive and Gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. Mol. Microbiol. **12:**333–334. http://dx.doi.org/10.1111/j.1365-2958.1994.tb01021.x.
54. **Hong H, Szabo G, Tamm LK.** 2006. Electrostatic couplings in OmpA ion-channel gating suggest a mechanism for pore opening. Nat. Chem. Biol. **2:**627–635. http://dx.doi.org/10.1038/nchembio827.
55. **Wang Y.** 2002. The function of OmpA in *Escherichia coli*. Biochem. Biophys. Res. Commun. **292:**396–401. http://dx.doi.org/10.1006/bbrc.2002.6657.
56. **Power ML, Ferrari BC, Littlefield-Wyer J, Gordon DM, Slade MB, Veal DA.** 2006. A naturally occurring novel allele of *Escherichia coli* outer membrane protein A reduces sensitivity to bacteriophage. Appl. Environ. Microbiol. **72:**7930–7932. http://dx.doi.org/10.1128/AEM.01040-06.
57. **Schweizer M, Henning U.** 1977. Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. J. Bacteriol. **129:**1651–1652.
58. **Orme R, Douglas CWI, Rimmer S, Webb M.** 2006. Proteomic analysis of *Escherichia coli* biofilms reveals the overexpression of the outer membrane protein OmpA. Proteomics **6:**4269–4277. http://dx.doi.org/10.1002/pmic.200600193.
59. **Reva ON, Weinel C, Weinel M, Bohm K, Stjepandic D, Hoheisel JD, Tummler B.** 2006. Functional genomics of stress response in *Pseudomonas putida* KT2440. J. Bacteriol. **188:**4079–4092. http://dx.doi.org/10.1128/JB.00101-06.
60. **Hallsworth JE, Heim S, Timmis KN.** 2003. Chaotropic solutes cause water stress in *Pseudomonas putida*. Environ. Microbiol. **5:**1270–1280. http://dx.doi.org/10.1111/j.1462-2920.2003.00478.x.
61. **Babich H, Stotzky G.** 1978. Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions. Appl. Environ. Microbiol. **36:**906–914.
62. **Silby MW, Levy SB.** 2004. Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: discovery of expressed sequences with novel genetic organization. J. Bacteriol. **186:**7411–7419. http://dx.doi.org/10.1128/JB.186.21.7411-7419.2004.
63. **Nishiyama E, Ohtsubo Y, Nagata Y, Tsuda M.** 2010. Identification of *Burkholderia multivorans* ATCC 17616 genes induced in soil environment by in vivo expression technology. Environ. Microbiol. **12:**2539–2558. http://dx.doi.org/10.1111/j.1462-2920.2010.02227.x.
64. **Boopathy R.** 2000. Factors limiting bioremediation technologies. Bioresour. Technol. **74:**63–67. http://dx.doi.org/10.1016/S0960-8524(99)00144-3.